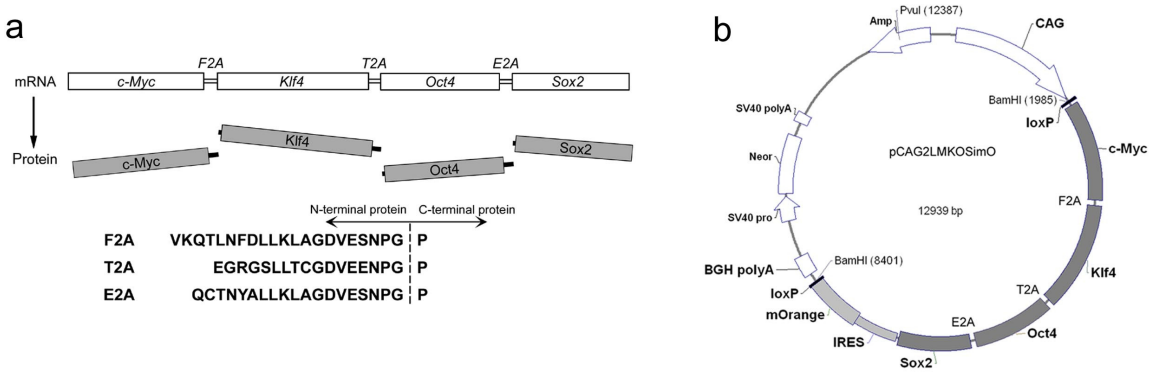
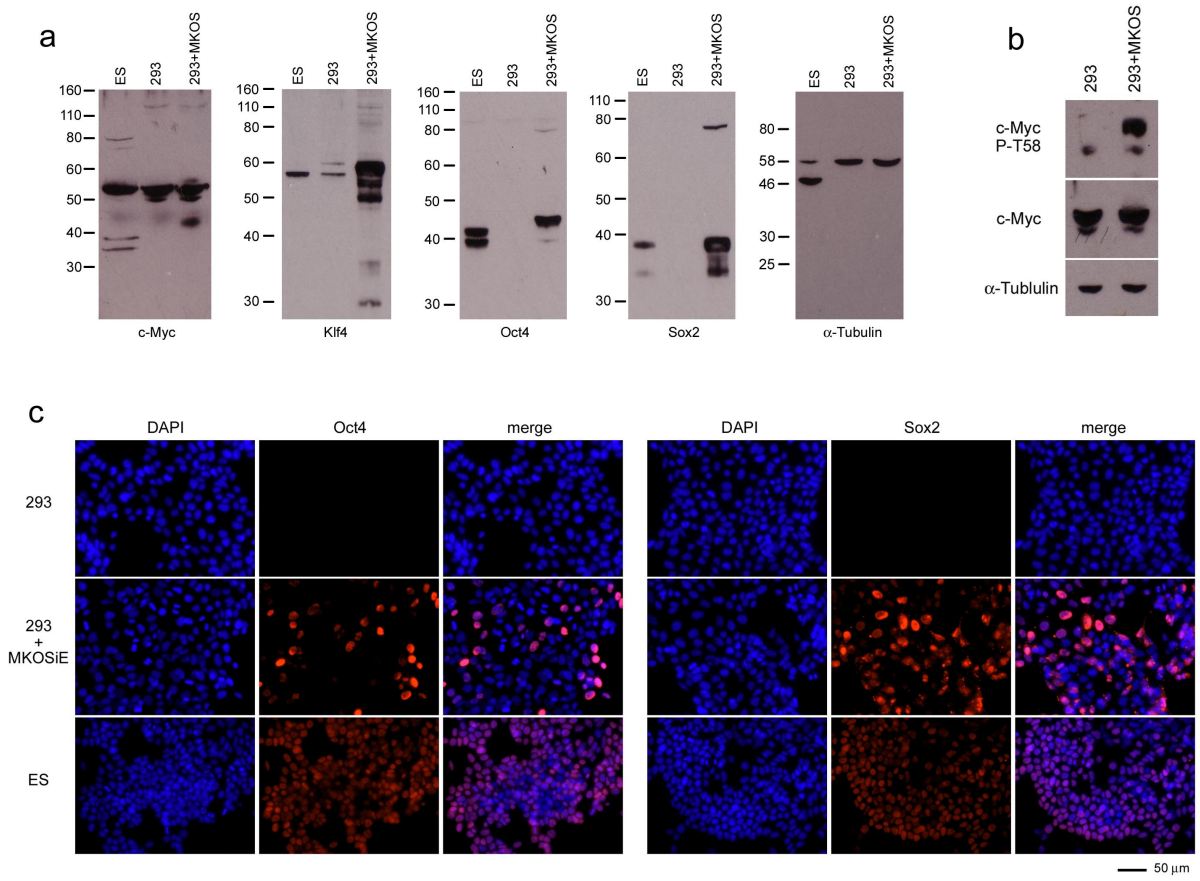
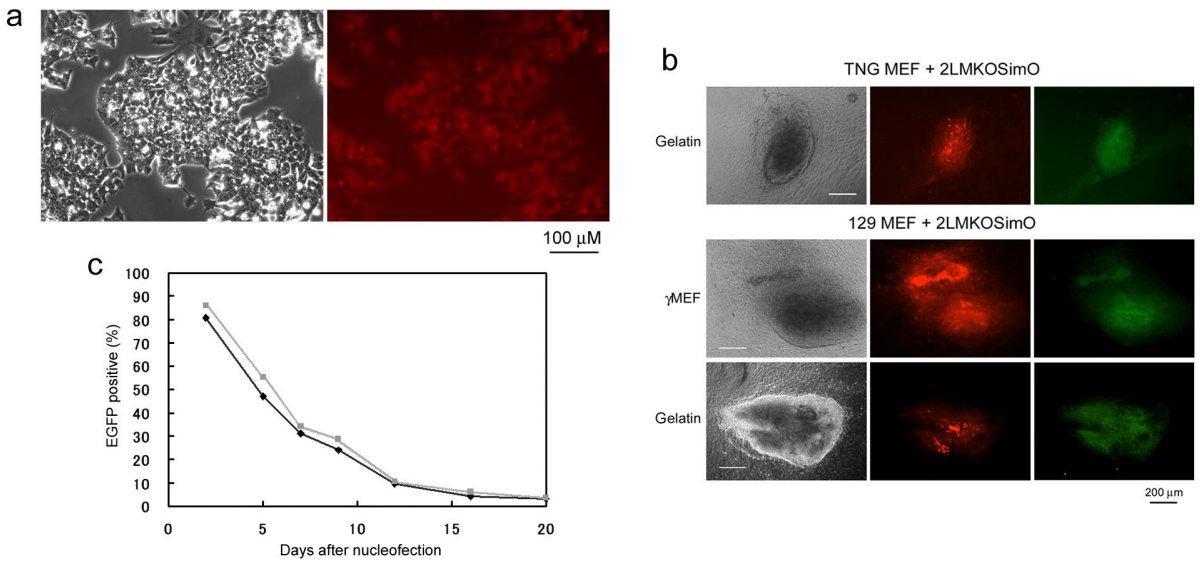


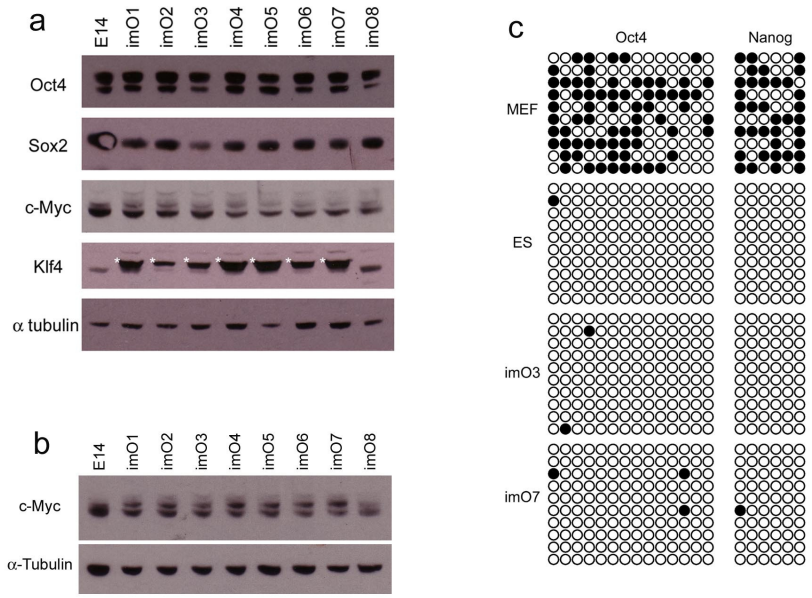
Supplementary Figure 1. Schematic of reprogramming cassette and a map of pCAG2LMKOSimO vector. a. The four reprogramming factors are translated from a single mRNA encoding *c-Myc*, *Klf4*, *Oct4* and *Sox2* linked with three different 2A sequences, *F2A*, *T2A* and *E2A.* The sequences of the 2A peptides and their 'skip' sites are shown. **b.** *c-Myc*, *Klf4*, *Oct4* and *Sox2* coding regions linked by three different 2A peptide sequences, *F2A*, *T2A* and *E2A* (reprogramming cassette) are transcribed from the CAG enhancer/promoter. The reprogramming cassette is followed by *ires mOrange*. The reprogramming cassette and *ires mOrange* are flanked by *loxP* sites. pCAG2LMKOSimO is linearized with PvuI before transfection.



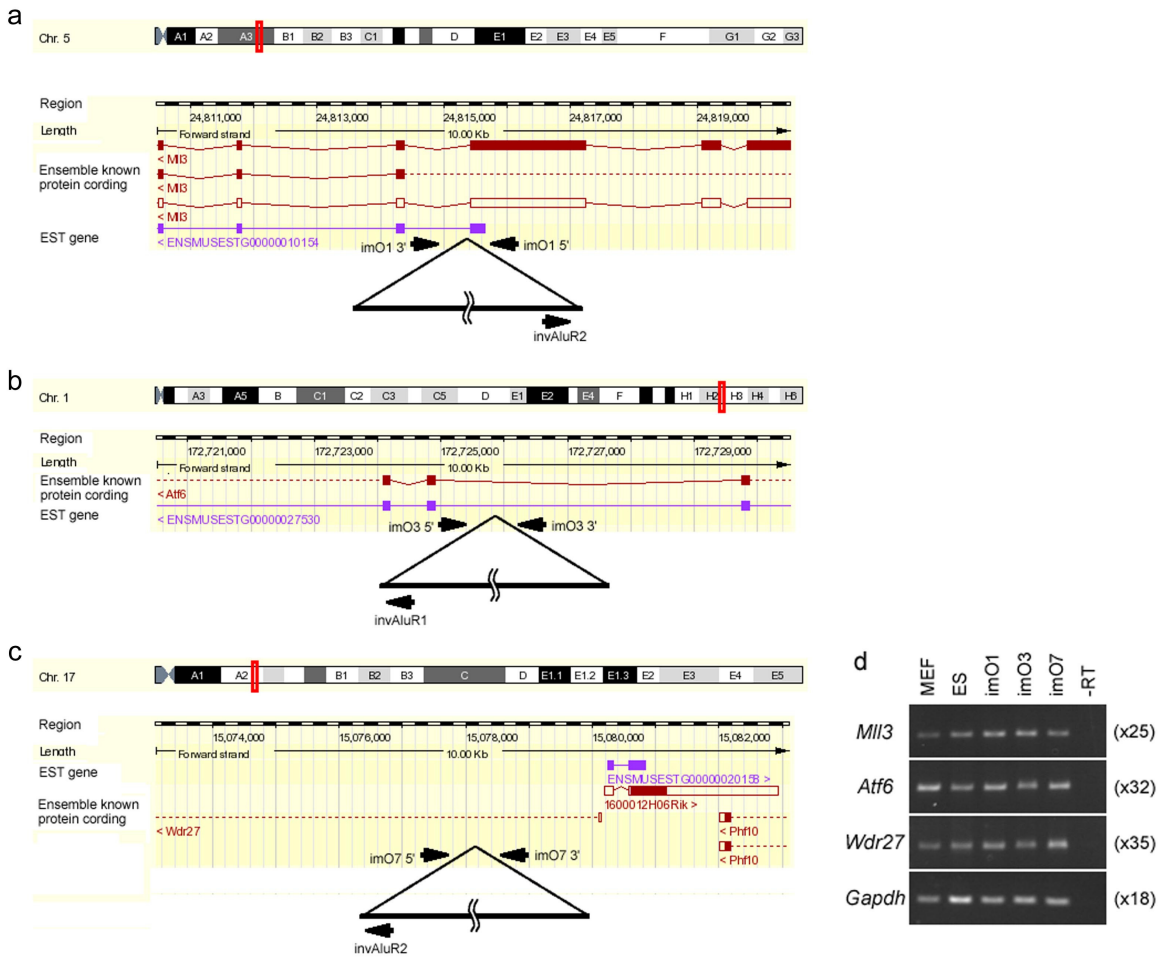
Supplementary Figure 2. Robust expression of reprogramming factors using a multiprotein expression vector. a. Immunoblot analysis of nuclear extracts of HEK293 cells 48 hrs post transfection with pCAGMKOSiE (293+MKOS). Nuclear extract of ES cells (ES) and HEK293 cells without nucleofection (293) are used to identify exogenous protein expression. An anti-α tubulin antibody is used as a loading control. **b**. Immunoblot analysis for phospho-T58 c-Myc in HEK293 with (293+MKOS) and without transfection (293). **c**. Immunofluorescence of HEK293 cells for Oct4 and Sox2 24 hrs post transfection of pCAGMKOSiE. ES cells and HEK293 cells provide positive and negative controls, respectively.



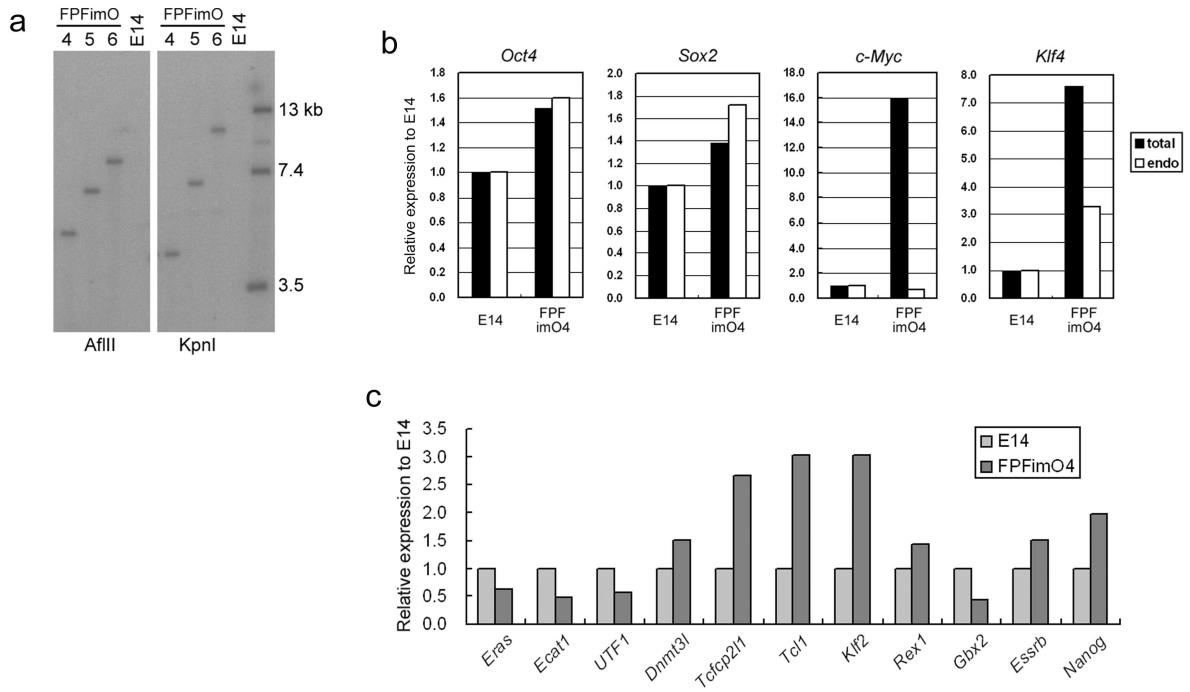
Supplementary Figure 3. Reprogramming with pCAG2LMKOSimO and estimation of stable transfection efficiency in MEF using nucleofection. a. mOrange positive ES cell like-colonies picked 20-30 days post nucleofection successfully grew on a gelatin-coated culture dish. **b.** TNG MEFs (top row) or non-genetically modified 129 MEFs (bottom two rows) were seeded on irradiated MEFs (γMEF) or gelatin (Gelatin) after nucleofection. Pictures represent Nanog-EGFP positive colonies from TNG MEFs and Nanog positive colonies (immunofluorescence) from 129 MEFs (green) with mOrange expression (red) 28 days post transfection. **c.** A vector that derives EGFP expression via the CAG enhancer/promoter was introduced into MEFs and the percentage of EGFP positive cells was monitored by FACS for 20 days after nucleofection without selection. The gray and black lines represent two independent experiments. Averages of EGFP positive cells were 86% and 3% of total cells at day 2 and day 20, respectively. We estimate stable integration occurred in 3.6% (3% of 86%) of transiently transfected cells.



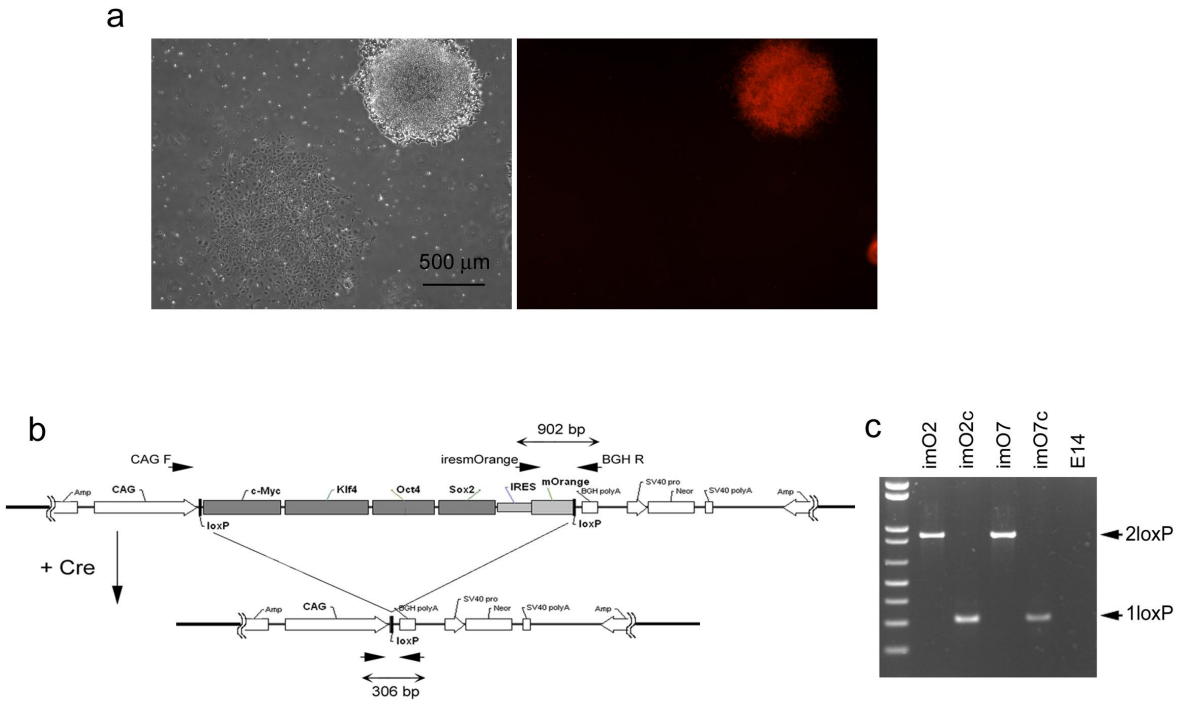
Supplementary Figure 4. Expression of reprogramming factor proteins and methylation states of *Oct4* and *Nanog* promoter in non-viral iPS cell lines. a. Immunoblot analysis of c-Myc, Klf4, Oct4 and Sox2. Asterisks indicate exogenous Klf4. **b.** Accumulation of exogenous c-Myc protein with higher molecular weight by a proteasome inhibitor**.** Cells were treated with 10 mM lactacystin for 3 hours before nuclear protein extraction. Exogenous c-Myc with higher molecular weight became more apparent in the iPS cell lines, suggesting degradation of exogenous c-Myc plays a role in keeping c-Myc protein at an ES cell level. **c.** Loss of DNA methylation of *Oct4* and *Nanog* promoters. The methylation state of the *Oct4* and *Nanog* promoters in MEFs, ES cells, and two reprogrammed cell lines, imO3 and imO7 was analyzed using bisulphite sequencing. Open circles indicate unmethylated and filled circles methylated CpG dinucleotides.



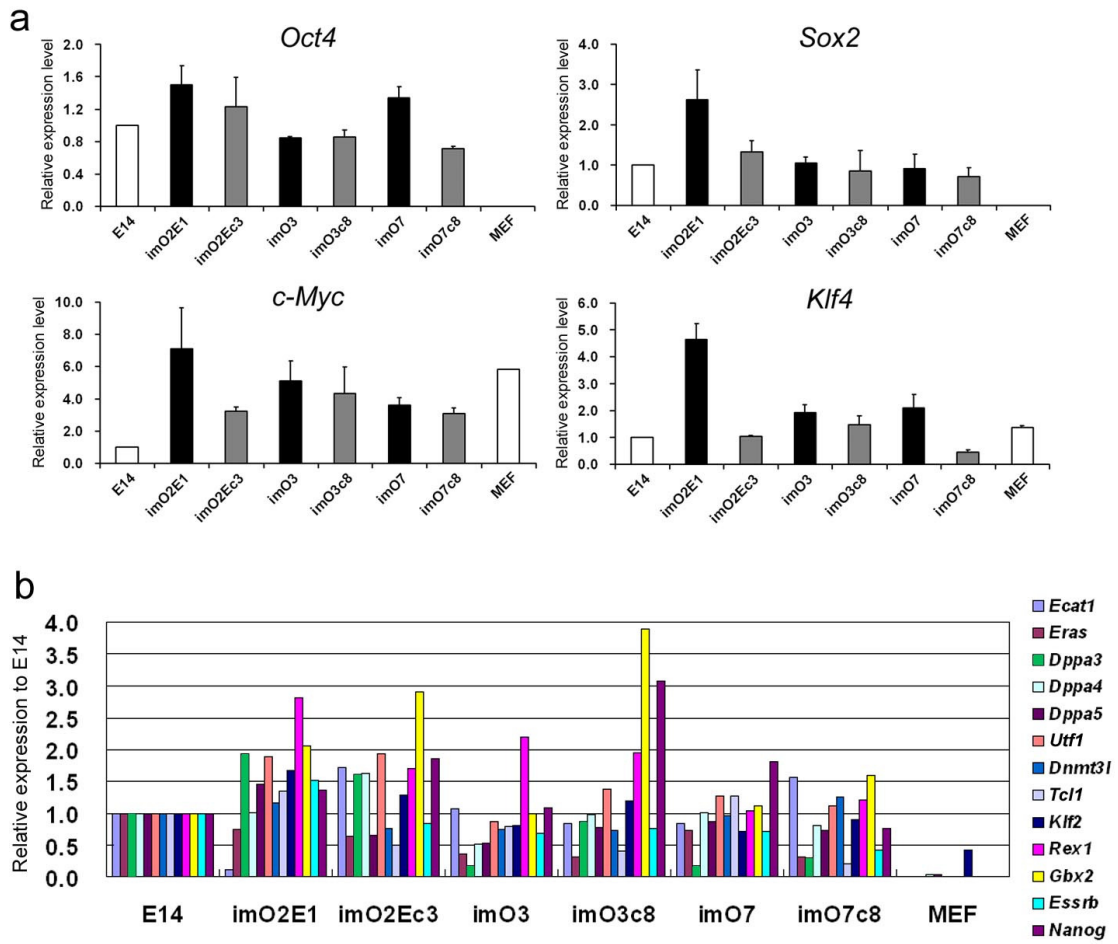
Supplementary Figure 5. Schematic presentation of vector integration sites and expression of the genes. a-c. Integration sites of imO1, imO3 and imO7 were found in chromosome 5 (a), chromosome 1 (b) and chromosome 17 (c), respectively. The red box in chromosome diagrams is enlarged below with the vector integration site indicated by black lines. Primers used for validation of the integration sites in Figure 2c are shown as black arrows. Diagrams and gene abbreviations were referred from Ensemble genomic databases. **d.** Expression of the genes in the integration sites. *Mll3*, *Atf6* and *Wdr27* expression is detectable in MEFs, ES cells and iPS cell clones, imO1, imO3, imO7 by RT-PCR. Numbers in parenthesis indicate cycle number of the PCR.



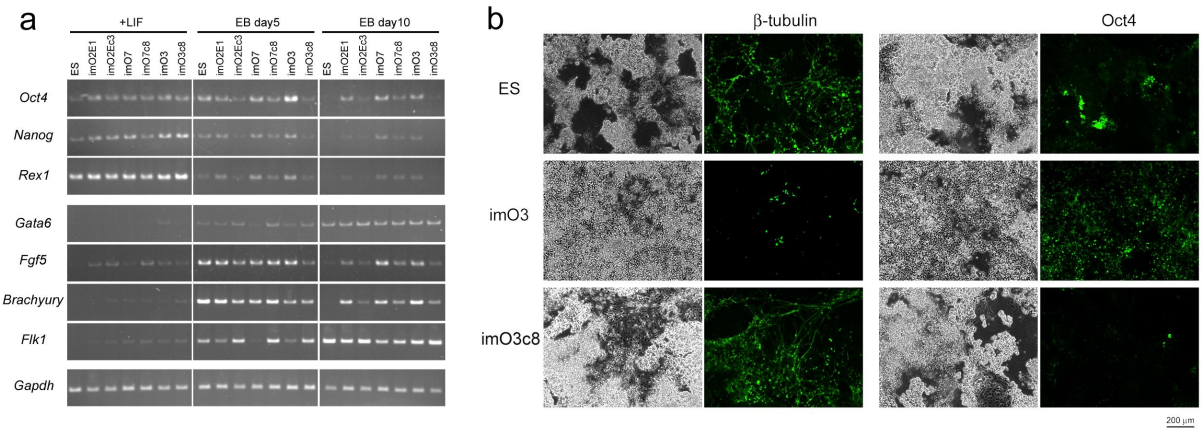
Supplementary Figure 6. Reprogramming of adult footpad fibroblasts. a. Southern blotting analysis for AflII (left panel) and KpnI (right panel) digested genome of FPFimO4-6, derived from adult footpad fibroblasts, using Orange probe. **b, c.** Quantitative PCR for total and endogenous *c-Myc*, *Klf4*, *Oct4* and *Sox2* expression (b) and pluripotent markers (c). Data is shown as relative expression to an ES cell line, E14Tg2a (E14) and represents one of two independent experiments. These data suggest single vector integration is enough to reprogram adult somatic cells.



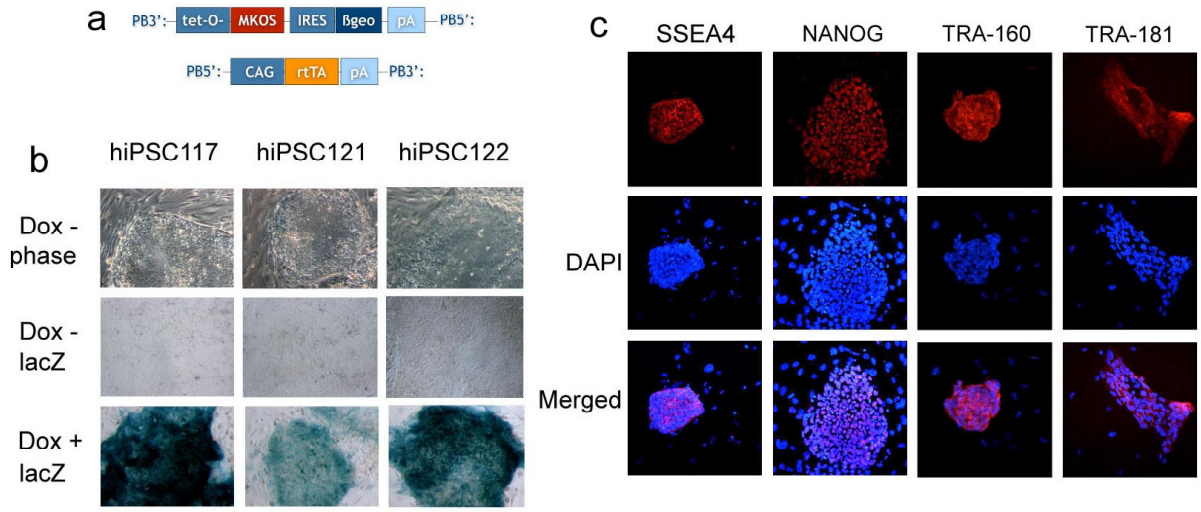
Supplementary Figure 7. Differentiation induced by Cre-mediated excision of the reprogramming cassette. a. Flat differentiated colonies with no mOrange expression and tight three-dimensional colonies with mOrange expression appeared seven days after *Cre* transfection. **b**. Diagram of the vector before and after Cre-mediated excision. Arrows indicates primers used to validate excision had occurred. **c**. Examples of genomic PCR before and after reprogramming cassette excision. Data represent one of the clones (imO2c and imO7c) generated from the cell lines, imO2 and imO7, respectively, by *Cre* transfection.



Supplementary Figure 8. Expression of reprogramming factors and pluripotent markers after Cre-excision. a. Quantitative PCR for total *c-Myc*, *Klf4*, *Oct4* and *Sox2* expression before (imO2E1, imO3, imO7; black) and after (imO2Ec3, imO3c8, imO7c8; gray) reprogramming cassette excision. Data is shown as relative expression to an ES cell line, E14Tg2a (E14). Error bars indicate the s.d. generated from triplicates. **b**. Quantitative PCR for pluripotency markers. Data represents one of two independent experiments.



Supplementary Figure 9. Efficient differentiation of reprogramming cassette-free iPS cell lines *in vitro*. **a**. Embryoid body (EB) differentiation. Cre-treated cell lines, imO2Ec3, imO3c8 and imO7c8 EBs showed similar down-regulation of pluripotent markers (*Oct4*, *Nanog*, *Rex1*), and up-regulation of endoderm (*Gata6*), ectoderm (*Fgf5*) and mesoderm (*Brachyury*, *Flk1*) markers to that of ES cells, while their parental cell lines, imO2E1, imO3 and imO7, differentiated less efficiently. Note up-regulation of *Fgf5* and *Brachyury* is transient in the EB differentiation. **b**. *In vitro* neural differentiation. Reprogramming cassette excised iPS cells, imO3c8, differentiated into β-tubulin positive neurons (green in left panels) as efficient as ES cells after 7 days in neural differentiation conditions. The parental iPS cell line, imO3, with an intact reprogramming cassette hardly generated neurons and maintained Oct4 expression (green in right panels).



Supplementary Figure 10. Non-viral reprogramming of human embryonic fibroblast with a single-vector reprogramming system combined with PiggyBac transposon. a. The structure of two PB transposons containing (i) a tetO promoter-driven 2A sequence-joint four reprogramming factors (MKOS) followed by *ß-geo* (top), and (ii) CAG enhancer/promoter-driven *rtTA* (bottom). **b.** Three clones, hiPSC117, hiPSC121 and hiPSC122, maintaining undifferentiated human ES cell-like morphology after withdrawal of doxycycline (Dox) (top panels). LacZ activity was hardly detectable in the absence of Dox in all three cell lines, suggesting the undifferentiated state of the cell lines was exogenous factors independent (middle and bottom panels). **c.** Expression of endogenous pluripotency markers (SSEA4, NANOG, TRA-160 and TRA-181) was observed in all three cell lines. Data shown from one of the cell lines, hiPSC122.

Supplementary Table 1. Estimation of reprogramming efficiency (%)

	Experiment No.	No. of expected stable transfectants[1]	Efficiency[2]	
			γMEF	Gelatin
TNG	1	295	-	2.03
	2	168	-	2.09
129	1	227	-	1.54
	2	134	4.48	1.49
	3	187	4.27	0.53
	4	216	3.24	2.78

1. Cell numbers which were expected to obtain stable integration were estimated from mOrange positive cell number at day 2 in Table 1, and stable transfection efficiency (3.6% of transient transfected cells as estimated in Supplementary Figure 2)
2. Reprogramming efficiency was estimated using the number of expected stable transfectants[1] and that of Nanog-GFP/Nanog positive colonies in Table 1. – indicates no data.

Supplementary Table 2. Summary of blastocyst injections

cell line	No. of transferred embryos	Chimeric embryos[1]					Pups	Live chimera (Chimerism[2])
		8.5 dpc	9.5 dpc	10.5 dpc	11.5 dpc	12.5 dpc		
imO7c8	52	-	-	-	-	-	28	5 (+++, +, +, +, +)
imO1c5	31	-	-	-	-	-	15	1 (+)
imO7Ec3	22	-	-	-	4/8	-	0	0
imO7Ec3+PD[3]	62	-	-	-	-	6/10	0	0
imO3c8	40	-	-	-	-	-	10	0
ImO3c8+PD[3]	39	-	-	-	-	-	8	0
imO3Ec5	39	-	-	-	-	-	10	0
imO3Ec5+PD[3]	96	8/10	7/8	4/5	-	6/8	12	1 (+)

[1] Number of GFP positive chimeric embryos / total embryos at indicated stages. -; not examined. Most of the chimeric embryos had high contribution as shown in Figure 3F.

[2] Chimerism was judged by coat color. +++ > 80%, + 10-30%.

[3] Cells were cultured in the presence of PD173074 for more than one week before injection. In other cases without the indication, cells were cultured for more than one week in the absence of PD173074.

Supplementary Table 3. Primers used for vector construction

Reprogramming cassette

EcoRI Koz 5'-Myc	gaattcaccatgcccctcaacgtgaactt
Myc-3' GSG F2A	tccacgtctcccgccaacttgagaaggtcaaaattcaaagtctgtttcacgccagaac ctgcaccagagtttcgaagct
F2A 5'-Klf4	cttgaatttgaccttctcaagttggcgggagacgtggagtccaacccagggcccatga ggcagccacctggc
Klf4-3' GSG T2A Xho	ctcgagtgggccaggattctcctcgacgtcaccgcatgttagcagacttcctctgccct ctccggagccaaagtgcctcttcatgtgta
Xho 5'-Oct4	ctcgagatggctggacacctggcttc
Oct4-3' GSG E2A	ttactttcaacatcgccagcgagtttcaacaaagcgtagttagtacattgacccgacc cgtttgaatgcatgggagagc
E2A 5'-Sox2	actacgctttgttgaaactcgctggcgatgttgaaagtaaccccggtcctatgtataa catgatggagac
Sox2-3' EcoRI Xho	ctcgagaattctcacatgtgcgacaggggca

loxP oligo

Kpn Bam loxP BamX F	cggatccataacttcgtatagcatacattatacgaagttatc
Kpn Bam loxP BamX R	gatcgataacttcgtataatgtatgctatacgaagttatggatccggtac

ires mOrange

Not IRES	gcggccgccctctccctcccccccccta
mOrange IRES	tgctcaccatggttgtggccatattatcat
IRES mOrange	ggccacaaccatggtgagcaagggcgagga
Xba Bam loxP mOrange	agtctagaggatccataacttcgtataatgtatgctatacgaagttatctacttgtac agctcgtccatg

Supplementary Table 4. Primers used for inverse PCR and Cre-mediated excision test

Inverse PCR

invAluF	gctgagtagtgcgcgagcaaa
invAluR1	gcgacacggaaatgttgaata
invAluR2	gccgcatagttaagccagtat
inv3TaqF1	tatctcagttcggtgtaggtcg
inv3TaqR	aagataccaggcgtttcccc

Integration site validation

Forward		Reverse	
imO1 5'	ttttttctcagggtgtgccag	invAluR2	gccgcatagttaagccagtat
		imO1 3'	ctcaaaccttcacagtgcca
imO3 5'	tgccgctccacaaatattga	invAluR1	gcgacacggaaatgttgaata
		imO3 3'	ggaggcatataaagcaatggc
imO7 5'	gggtactggaattggagctc	invAluR2	gccgcatagttaagccagtat
		imO7 3'	accatcattagtcagctcccct

Concatemer test

Forward		Reverse	
inv3TaqF1	tatctcagttcggtgtaggtcg	invAluR2	gccgcatagttaagccagtat

Cre-mediated excision test

Forward		Reverse	
CAG F	ctctgctaaccatgttcatgc	BGH R	tagaaggcacagtcgagg
IRESmOrange	ggccacaaccatggtgagcaagggcgagga		

Supplementary Table 5. Gene specific primers

Quantitative PCR

Genes Forward Reverse

Total

c-Myc	MycT F	cctagtgctgcatgaggaga	MycT R	tcttcctcatcttcttgctcttc
Klf4	Klf4T F	cgggaagggagaagacact	Klf4T R	gagttcctcacgccaacg
Oct4	Oct4T F	gttggagaaggtggaaccaa	Oct4T R	ctccttctgcagggctttc
Sox2	Sox2T F	acagctacgcgcacatga	Sox2T R	ggtagcccagctgctcct

Endogenous

c-Myc	c-Myc F	tcaagcagacgagcacaagc	c-Myc R	tacagtcccaaagccccagc
Klf4	Klf4 F	ggcgagaaaccttaccactgt	Klf4 R	tactgaactctctctcctggca
Oct4	Oct4-906F	ccaacgagaagagtatgaggc	Oct4UTR R	gtgcttttaatccctcctcag
Sox2	Sox2-3 F	tctgtggtcaagtccgaggc	Sox2UTR R	ttctccagttcgcagtccag

Ecat1	Ecat1 FT	ggcgagctgagatttggata	Ecat1 RT	ccagcctccagagcctctat
Eras	Eras FT	gcccctcatcagactgctac	Eras RT	gcagctcaaggaagaggtgt
Dppa3	Dppa3 FT	gatgcacaacgatccagattt	Dppa3 RT	tggaaattagaacgtacatactccaa
Dppa4	Dppa4 FT	aagggctttcccagaacaat	Dppa4 RT	tccagaggaactgtcacctca
Dppa5	Dppa5 FT	attcgggctaaatggatgc	Dppa5 RT	tagctccagggtcttcatgg
Utf1	Utf1 FT	gtccctctccgcgttagc	Utf1 RT	ggggcaggttcgtcattt
Dnmt3l	Dnmt3l FT	caactacccgcttccttcag	Dnmt3l RT	cccgcatagcattctggta
Tcl1	Tcl1F	gcttcctctctgggtgttca	Tcl1R	cccacacattccctttcaac
Klf2	Klf2 F	ctaaaggcgcatctgcgta	Klf2 R	tagtggcgggtaagctcgt
Rex1	Rex1F	ggaagaaatgctgaaggtggagac	Rex1R	agtccccatccccttcaatagc
Gbx2	Gbx2 F	gctgctcgctttctctgc	Gbx2 R	gctgtaatccacatcgctctc
Essrb	Esrrb F	tggcaggcaaggatgacaga	Esrrb R	tttacatgagggccgtggga
Nanog	NanogQT F	cctccagcagatgcaagaa	NanogQT R	gcttgcacttcatcctttgg
Tbp	Tbp FT	ggggagctgtgatgtgaagt	Tbp RT	ccaggaaataattctggctca

Embryoid body PCR

Genes Forward Reverse

Oct4	Oct4 F	ccaacgagaagagtatgaggc	Oct4 R	agagcagtgacgggaacagag
Nanog	Nanog F	gtgcatatactctctccttccc	Nanog R	agctaccctcaaactcctggt
Rex1	Rex1 F	ggaagaaatgctgaaggtggagac	Rex1 R	agtccccatccccttcaatagc

Fgf5	Fgf5 F2	ttgcgacccaggagcttaat	Fgf5 R2	ctacgcctctttattgcagc
Brachyury	T F2	ccaaggacagagagacggct	T R2	agtaggcatgttccaagggc
Gata6	Gata6 F	cccacttctgtgttcccaattg	Gata6 R	ttggtcacgtggtacaggcg
Flk1	Flk1 F	gcttgctccttcctcatctc	Flk1 R	ccatcaggaagccacaaagc
Gapdh	Gapdh F	gtgttcctacccccaatgtg	Gapdh R	gtcattgagagcaatgccag

Supplementary Methods

Cell culture and blastocyst injection

MEFs from 13.5-14.5 dpc 129 mice embryos were generated following a standard method. To isolate fibroblasts from adult mouse footpads, finely chopped footpad skin of 6-8 weeks old 129 mice were incubated in 0.25% trypsin, 1mM EDTA, 100 μg/ml DNase in PBS at 37 °C. After 15 minutes the trypsin solution was collected into a tube with GMEM containing 10% FCS through a fine mesh to remove debris. The skin was trypsinized two more times in the same way. All isolated cells were combined and cultured in GMEM containing 10% FCS, penicillin/streptomycin, L-glutamine, β-mercaptoethanol, non-essential amino acids, and human basic FGF (10 ng/ml). The footpad fibroblasts (FPFs) were used for experiments within 4-8 passages. MEFs from C57BL/6 strain TNG mice [23] (TNG MEFs) were kindly provided by I. Chambers. MEFs and HEK293 cells were cultured in the same medium as for FPFs without basic FGF. Leukemia inhibiting factor (LIF) (1,000 U/ml) was added for iPS cell derivation and maintenance. ES cell-like colonies derived from 129 MEFs were picked 4 weeks after nucleofection, trypsinised with 0.25% trypsin, 0.1% EDTA in PBS, and seeded on γMEFs or directly on gelatin in 4 well plates as the first passage. A wild type ES cell line, E14Tg2a, and established iPS cells were passaged on gelatin-coated dishes every 2-3 days. Cell lines, imO2Ec3, imO3Ec5 and imO7Ec3, were generated by excising the reprogramming cassette from imO2E1, imO3E1 and imO7E1, respectively, which has randomly integrated CAG-EGFP cassette. Embryoid body formation and neural differentiation was performed and the resulting cells were used for RT-PCR and immunofluorescence as described before[26,27]. Chimeras were produced by microinjection into C57BL/6 blastocysts. Chimerism of embryos was analyzed at 8.5-12.5 dpc by observing GFP expression. Genital ridges from 12.5 dpc embryos were stained with anti-Oct4 antibody (N-19, Santa Cruz) and observed with a confocal microscope (Leica TSC SP2). Cre-treated cell lines were cultured for more than one week in the absence of PD173074 before RNA extraction, teratoma formation assay and blastocyst injections.

Immunoblotting

Nuclear extracts were prepared as described before[26]. Transfected HEK293 cells were harvested 48 hrs post transfection. Antibodies against c-Myc (N-262), Klf4 (H-180) Oct4 (N-19), Sox2 (Y-17) and α-tubulin (B-7) were purchased from Santa Cruz, and an anti-phospho-T58 c-Myc antibody (ab28842) was purchased from Abcam.

Inverse PCR

Genomic DNA extracted from imO1, imO3, and imO7 was digested with AluI, SpeI or TaqI.

After self-ligation for 18 hours at 16 °C, the DNA was used as PCR templates for primers in Supplementary Table 4. Amplified fragments were purified, cloned into pCRII-TOPO vector (Invitrogen) and sequenced. The integration sites of imO1 and imO7 were identified with invAluF/invAluR2 primers in AluI digested genome and SpeI digested genome, respectively. The integration site of imO3 was identified with invAluF/invAluR1 primers in AluI digested genome. inv3TaqF1/inv3TaqR primers identified concatemers in the TaqI-digested imO1 and imO3 genomes. The integration site and concatemer were verified using specific primers listed in Supplementary Table 4.

Bisulfite sequencing

Bisulfite conversion was performed using Imprint DNA Modification Kit (Sigma) following manufacturer's instruction. The promoter regions of Oct4 and Nanog are amplified with specific primers[1], and the products were cloned into pCRII-TOPO vector (Invitrogen). Ten randomly chosen clones were sequenced from each cell line for each gene.

Human embryonic fibroblast (HEF) isolation

Twelve week-old abortion material washed in PBS, macerated and treated thrice with TrypLE, 0.1% EDTA. The suspension was plated on untreated dishes in HEF media (DMEM, 15% human serum, 10 ng/ml bFGF, penicillin-streptomycin, glutamax, β-mercaptoethanol, non-essential amino acids) and grown for 3 days at 37 °C, 5% CO_2. Then the primary cells were passaged once for expansion and frozen in aliquots (p2).

PB transfection and cell culture

HEFs were grown to 90% confluency (36-48 hrs at 37°C, 5% CO_2) and seeded at a density of 3.2 and 6.4x10^4 cells/10 cm^2 in 6-well plates containing HEF medium (DMEM, 15% human serum, 10 ng/ml bFGF, penicillin-streptomycin, glutamax, β-mercaptoethanol, non-essential amino acids). After 18-24 hrs growth, the cells were transfected with 100 ng or 250 ng of transposon delivering the 2A sequence linked reprogramming factors, the PB embedded CAG-rtTA-pA cassette and 100 ng of pCyL43 PB transposase plasmid[15] (normalized to 2 μg total DNA with empty pBluescriptKS+). To generate transfection complexs 2 μg total DNA was diluted to 100 μl with DMEM, mixed with FugeneHD (Roche) at a Fugene:DNA ratio of 8 μl:2 μg, incubated at room temperature for 15-20 min, and added to the media. 24 hours post-transfection 1.5 mg/ml doxycycline was added to the medium and kept for 32 days. 48 hrs post-transfection the HEF medium was changed to ES medium (HEScGRO). Cells were fed daily until analyzed. Colonies were picked and passaged using mechanical dissociation.